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14. ABSTRACT

During the second reporting period, we characterized the AR-microtubule interaction and concluded that AR directly associates with the microtubules. We found that the microtubule-binding activity highly correlates with the cytoplasmic localization of AR in the absence of androgen. By attaching microtubule-associated sequences (MTAS) to AR-V7 and ARv567es, we were able to trap these AR-Vs in the cytoplasm. These results suggest that the microtubules play an important role in controlling the intracellular localization of AR, and that the constitutive nuclear localization of AR-V7 and ARv567es is due to escaping of the microtubule-mediated cytoplasmic retention. Finally, we have further developed and validated a whole-blood assay for the detection of both AR-V7 and ARv567es, without the need for isolation or enrichment for circulating tumor cells.

15. SUBJECT TERMS
Castration-resistant prostate cancer; docetaxel; cabazitaxel; chemotherapy; androgen receptor splice variants; microtubule; ligand-binding domain; microtubule-associated sequence

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1. Introduction

Docetaxel-based chemotherapy is established as a first-line treatment and standard of care for patients with metastatic castration-resistant prostate cancer (mCRPC). However, half of the patients do not respond to treatment and those do respond eventually become refractory. A better understanding of the resistance mechanisms to taxane chemotherapy is both urgent and clinical significant, as taxanes (docetaxel and cabazitaxel) are being used in various clinical settings. Sustained signaling through the androgen receptor (AR) has been established as a hallmark of CRPC. Recently, several alternative splicing variants of AR (AR-Vs) that lack the ligand-binding domain (LBD) have been identified. Preliminary studies conducted in our laboratory showed increased expression of AR-Vs (AR-V7 and AR^{v567es}) rendered prostate cancer cells less responsive to taxane drugs. The objective of this application is to test the hypothesis that constitutively active AR-Vs are associated with resistance to taxane chemotherapy in CRPC.

2. Keywords

Castration-resistant prostate cancer; docetaxel; cabazitaxel; chemotherapy; androgen receptor splice variants; microtubule; ligand-binding domain; microtubule-associated sequence

3. Accomplishments

What are the major goals/tasks of this project?

Major Task 1: To determine if ectopic expression of caARVs in tumor xenografts reduces the sensitivity to taxanes.
Major Task 2: To determine if knockdown of caARV sensitizes LNCaP95 and LuCaP 136 xenografts to taxanes.
Major Task 3: To identify the microtubule-associated sequence (MTAS) on AR.
Milestone: Identify the sequence of AR that is involved in microtubule-binding. Publish 1 peer-reviewed paper.
Major Task 4: To conduct a clinical study to evaluate the correlation between caARVs expression and response to taxane chemotherapy in patients treated for mCRPC.

What was accomplished under these goals?

Expanded Task 3

To determine if AR interacts directly with the microtubules. COS-7 cells transfected with flag-tagged AR were lysed in a high stringency buffer (RIPA buffer) and AR was immunoprecipitated with the anti-Flag antibody. Western analyses were carried out to validate the purity of AR and confirmed that recovered AR was free of contamination of chaperones (HSP90 and HSP70) and tubulin (Fig. 1, left). Subsequently, AR was combined with assembled microtubules in an *in vitro* microtubule binding assay and

subjected to co-immunoprecipitation with the anti-Flag antibody (Fig. 1, right). The result suggests that AR interacts directly with the microtubules.

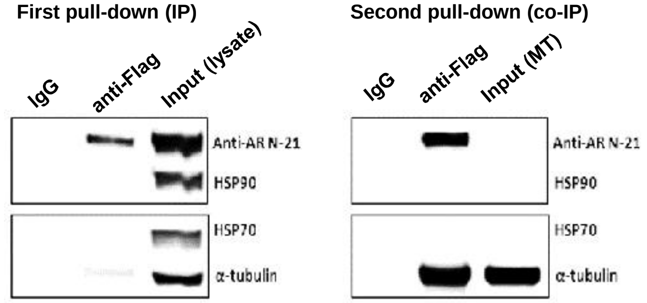

Fig. 1. AR binds directly to microtubules. Left, COS7 cells expressing flag-tagged AR were lysed in RIPA buffer and the lysates were subjected to pull-down assay with the anti-Flag antibody. Western analyses confirmed that AR recovered from the first pull-down was free of chaperones (HSP90 and HSP70) and tubulin. **Right**, AR was subjected to an *in vitro* microtubule binding assay (Cytoskeleton, Cat. # BK029) with assembled microtubules and subjected to co-IP (second pull-down) with the anti-Flag antibody.

To functional characterize the microtubule-associated sequences (MTAS) on AR. In the previous report, we have identified two regions in the AR ligand-binding domain containing MTAS. The two regions appear to be functionally redundant, as either one was able to confer microtubule binding. We have also demonstrated that the microtubule-binding activity highly correlates with the cytoplasmic localization of AR in the absence of androgen, suggesting that microtubule-binding confers cytoplasmic retention for unliganded AR. To test the functional significance of the MTAS, we constructed fusion proteins containing AR-V7 or AR^{v567es} and an MTAS, and examined the localization of the fusion proteins. As shown in Fig. 2, attachment of the MTAS changed the intracellular localization of both AR-V7 and AR^{v567es} from predominantly nuclear to predominantly cytoplasmic. This result suggests that the constitutive nuclear localization of AR-V7 and AR^{v567es} is due to escaping of the microtubule-mediated cytoplasmic retention.

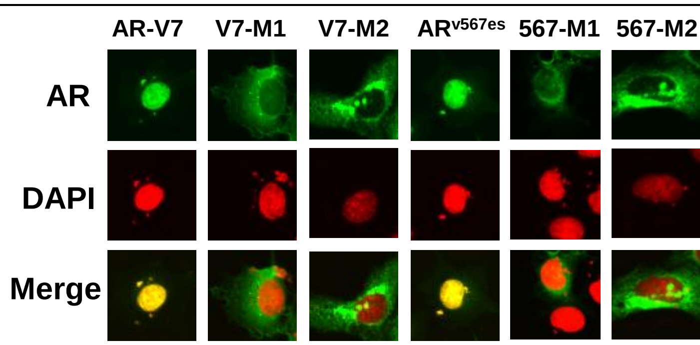

Fig. 2. Microtubule association traps AR-Vs in the cytoplasm. The previously identified MTAS1 (a.a 732-744, M1) and MTAS2 (a.a. 815-880, M2) were fused with AR-V7 and AR^{v567es}, and transfected into COS7 cells. The cells were cultured on cover slips in androgen-free media. The localization of the fusion proteins was analyzed by immunofluorescence with an antibody recognizes the N-terminus of AR. The nuclei were stained with DAPI (pseudocolored red for better visualization).

Summary for Task 3: we have completed the proposed experiment listed under Major Task 3. We also extended Task 3 with additional experiments to achieve a better understanding of the role of microtubule in regulating the intracellular localization of AR.

Task 4: To conduct a clinical study to evaluate the correlation between AR-Vs expression and response to taxane chemotherapy in patients treated for mCRPC.

Evaluation of protocols for detecting AR-Vs in the blood. In the previous report, we described the PAXgene assay for detection of AR-Vs in the blood. We further tested the specificity of this approach in two patient cohorts. The first is a heavily treated mCRPC cohort and the second is comprised of patients who have received radical prostatectomy with undetectable PSA (<0.01 ng/mL). As shown in Table 1, AR-V7 was expressed in the majority of the mCRPC cohort, but not detected in the post-radical prostatectomy (post-RP) cohort. Similarly, AR^{v567es} was detectable in more than 30% of the sample from the mCRPC cohort, but undetectable in the post-RP cohort. These results suggest that the PAXgene assay specifically detects AR-Vs expressed in cancer cells.

Table 1. Detection of AR transcripts by the PAXgene assay.

	Post-RP	**mCRPC**	***P* value***
Age at sampling	65.05 (range 53-76)	68.52 (range 47-86)	0.1559
AR-FL+	17/21 (80.95%)	69/73 (94.52%)	0.0712
AR-V7+	0/21 (0%)	50/73 (68.49%)	<0.0001
AR^{v567es} +	0/21 (0%)	23/73 (31.51%)	0.0014
AR-V+	0/21 (0%)	53/73 (72.60%)	<0.0001
AR-V7+, AR^{v567es}+	0/21 (0%)	20/73 (27.40%)	0.0051

*For dichotomous variables, *P*-values were calculated by Fisher's Exact Test. For Age at sampling, *P*-value was by the *Student's* t-test.

This study has been published on the Journal of Urology. Please see the attached reprint for additional data.

Summary for Major Task 4: we have developed and validated the sample collection procedure and whole blood-based assay for detecting the expression of AR-V7 and AR^{v567es} in patients. Patient selection and sample collection are ongoing.

What opportunities for training and professional development has the project provided?

Nothing to report.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

We are still in the process of identifying a candidate with strong skills in animal studies. In the next reporting period, we will start the animal studies and continue the clinical study under Major Task 4.

<u>Citations (published journal articles):</u>

Xichun Liu, Elisa Ledet, Dongying Li, Ary Dotiwala, Allie Steinberger, Jianzhuo Li, Yanfeng Qi, Yan Dong, Jonathan Silberstein, Benjamin Lee, Oliver Sartor, and Haitao

Zhang. A Whole Blood-Based Detection Assay for AR-V7 and AR^{v567es} in Patients with Advanced Prostate Cancer. Journal of Urology 2016;196:1758-1763.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?

Constitutively active AR-Vs have been associated with resistance to hormonal therapies. With the support of this grant, we have developed and validate a whole-blood assay for the detection of AR-V7 and AR^{v567es}, without the need for isolation or enrichment for circulating tumor cells. Due to the sensitivity, specificity, practicality, and cost effectiveness, this assay can be easily implemented, allowing more patients to benefit from this assay. The identification and functional characterization of the microtubule-binding sequence in the AR-LBD could deepen our understanding of the regulatory network controlling the intracellular localization and trafficking of AR, as well as further our understanding of the constitutive nuclear localization of AR-Vs.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. Changes/Problems

Dr. Dongying Li joined the project and worked on the validation of the PAXgene assay.

6. Products

Publications, conference papers, and presentations

- **Journal publications.** The following paper was published:

  Xichun Liu, Elisa Ledet, Dongying Li, Ary Dotiwala, Allie Steinberger, Jianzhuo Li, Yanfeng Qi, Yan Dong, Jonathan Silberstein, Benjamin Lee, Oliver Sartor, and Haitao Zhang. A Whole Blood-Based Detection Assay for AR-V7 and AR^{v567es} in Patients with Advanced Prostate Cancer. Journal of Urology 2016;196:1758-1763. Status: Published; Acknowledgement of federal support: yes.

- **Books or other non-periodical, one-time publications.** Nothing to report.

- **Other publications, conference papers, and presentations.** Presentations:

Jianzhuo Li, Guanyi Zhang, Hee-Won Park, **Haitao Zhang**. Mechanism of the anterograde transport of the androgen receptor. AACR Annual Meeting 2016, New Orleans, LA, April 16-20.

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Nothing to report.

Others

Nothing to report.

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name	Haitao Zhang	Guanyi Zhang	Dongying LI	Elisa Ledet	Brian Lewis
Project role	PI	Technician	Postdoctoral Fellow	Study coordinator	Co-investigator
Researcher Identifier (ORCID ID)	0000-0002-5969-1024	N.A.	N.A.	N.A.	N.A.
Nearest person month worked	3	9	2	0.5	0.5
Contribution to project	Project design; data analysis; Study coordination; presentation; manuscript writing; report	Characterization of MTAS	Validate PAXgene assay in the cancer-free cohort	Clinical study coordinator	Patient recruitment and consent
Funding support	DOD-PCRP, American Cancer Society	DOD-PCRP American Cancer Society	DOD-PCRP, American Cancer Society	DOD-Postdoctoral Fellowship	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. Special Reporting Requirements: not applicable.

9. Appendices

Xichun Liu, Elisa Ledet, Dongying Li, Ary Dotiwala, Allie Steinberger, Jianzhuo Li, Yanfeng Qi, Yan Dong, Jonathan Silberstein, Benjamin Lee, Oliver Sartor, and Haitao Zhang. A Whole Blood-Based Detection Assay for AR-V7 and AR^{v567es} in Patients with Advanced Prostate Cancer. Journal of Urology 2016;196:1758-1763.

A Whole Blood Assay for AR-V7 and AR^{v567es} in Patients with Prostate Cancer

Xichun Liu,* Elisa Ledet,* Dongying Li, Ary Dotiwala, Allie Steinberger, Allison Feibus, Jianzhuo Li, Yanfeng Qi, Jonathan Silberstein, Benjamin Lee, Yan Dong, Oliver Sartor and Haitao Zhang†

From the Departments of Pathology (XL, DL, JL, HZ), Structural and Cellular Biology (YQ, YD), Medicine (EL, AD, AS, OS) and Urology (EL, AD, AS, AF, JS, BL, OS) and Tulane Cancer Center (XL, EL, DL, AD, AS, JL, YQ, YD, OS, HZ), Tulane University School of Medicine, New Orleans, Louisiana

Abbreviations and Acronyms

AR-FL = full-length androgen receptor

CRPC = castration resistant prostate cancer

CTC = circulating tumor cell

EMT = epithelial-to-mesenchymal transition

EpCAM = epithelial cell adhesion molecule

mCRPC = metastatic castration resistant prostate cancer

PCR = polymerase chain reaction

PSA = prostate specific antigen

RP = radical prostatectomy

Purpose: Most prostate cancer mortality can be attributed to metastatic castration resistant prostate cancer, an advanced stage that remains incurable despite recent advances. The AR (androgen receptor) signaling axis remains active in castration resistant prostate cancer. Recent studies suggest that expression of the AR-V (AR splice variant) AR-V7 may underlie resistance to abiraterone and enzalutamide. However, controversy exists over the optimal assay. Our objective was to develop a fast and sensitive assay for AR-Vs in patients.

Materials and Methods: Two approaches were assessed in this study. The first approach was based on depletion of leukocytes and the second one used RNA purified directly from whole blood preserved in PAXgene® tubes. Transcript expression was analyzed by quantitative reverse transcription-polymerase chain reaction.

Results: Through a side-by-side comparison we found that the whole blood approach was suitable to detect AR-Vs. The specificity of the assay was corroborated in a cancer-free cohort. Using the PAXgene assay samples from a cohort of 46 patients with castration resistant prostate cancer were analyzed. Overall, AR-V7 and AR^{v567es} were detected in 67.53% and 29.87% of samples, respectively. Statistical analysis revealed a strong association of AR-V positivity with a history of second line hormonal therapies.

Conclusions: To our knowledge this is the first study to demonstrate that PAXgene preserved whole blood can be used to obtain clinically relevant information regarding the expression of 2 AR-Vs. These data on a castration resistant prostate cancer cohort support a role for AR-Vs in resistance to therapies targeting the AR ligand-binding domain.

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No direct or indirect commercial incentive associated with publishing this article.

The corresponding author certifies that, when applicable, a statement(s) has been included in the manuscript documenting institutional review board, ethics committee or ethical review board study approval; principles of Helsinki Declaration were followed in lieu of formal ethics committee approval; institutional animal care and use committee approval; all human subjects provided written informed consent with guarantees of confidentiality; IRB approved protocol number; animal approved project number.

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* Equal study contribution.

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See Editorial on page 1606.

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Key Words: prostatic neoplasms; receptors, androgen; blood; biomarkers, tumor; protein isoforms

PROSTATE cancer is the second leading cause of cancer mortality in men in the United States. Most disease related deaths can be attributed to mCRPC, which is marked by increasing serum PSA levels approximately 16 months after initial androgen deprivation therapy. Despite recent advances mCRPC remains the most critical challenge in the clinical management of prostate cancer.

It is well accepted that the AR signaling axis has a critical role in CRPC. Through a number of ligand dependent and independent mechanisms cancer cells adapt to low circulating androgens and maintain activation of AR. Particularly, a number of AR-Vs that are devoid of a functional ligand-binding domain have been identified.[1–4] Two major variants, AR-V7 and AR^{v567es}, have been shown to be capable of regulating target gene expression independent of AR-FL.[2–5] Recent studies suggest that the expression of these AR-Vs underlies resistance to second line hormonal therapies.[6,7]

CTCs are shed from solid tumors into the circulation. A number of CTC detection technologies have been developed in recent years. To date the most widely adopted is surface marker based CTC capturing, which relies on cell surface antigens such as EpCAM, cytokeratin, PSMA (prostate specific membrane antigen) or a combination of these markers.[8] Alternatively, CTCs can be enriched by depleting hematopoietic cells. For this purpose CD45, which is expressed on the surface of all leukocytes and their progenitors,[9] is commonly used.[10,11] Yet other studies have demonstrated the validity of using whole blood derived RNA from patients for reverse transcription-PCR analyses without CTC selection or enrichment.[12–14] The results of these series, which focused on genes highly expressed in prostate cancer, were clinically relevant and highly concordant with CTC enumeration analyses.[13,14]

In this study we sought to detect the expression of AR-V7 and AR^{v567es} in the circulation. We evaluated the CD45 based negative selection approach and the whole blood approach. Based on the results we chose the whole blood approach and analyzed blood samples obtained from a cohort of patients with CRPC.

MATERIALS AND METHODS

RNA Extraction from Whole Blood

From each patient 5 ml blood were collected into 2 PAXgene Blood RNA Tubes. The tubes were gently inverted and incubated at room temperature for 2 to 24 hours or stored at −20C before processing. Prior to RNA isolation frozen samples were brought to room temperature for 2 hours and centrifuged at 3,000 × gravity for 10 minutes. RNA isolation was performed using the PAXgene Blood RNA Kit.

Leukocyte Depletion

CTC enrichment was performed using a 2-step procedure as described[11] with modifications.

Red Blood Cells Lysis. Patient blood (10 ml) was collected in sodium citrate tubes (BD™) for immediate processing. Red blood cells were removed by adding lysis buffer composed of 154 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA (ethylenediaminetetraacetic acid) in 25 ml buffer per ml blood. After 5 minutes at room temperature the remaining cells were collected by centrifugation at 300 × gravity for 5 minutes. The pellet was washed twice with the labeling buffer (phosphate buffered saline with 2 mM EDTA and 0.5% bovine serum albumin, Ca^{2+}/Mg^{2+} free) and resuspended. Cells were counted and the concentration was adjusted to 1×10^8 cells per ml.

Leukocyte Removal. Following red blood cell lysis cell suspension was transferred to a round-bottom tube (BD). For every 10^8 cells 200 µl FcR Blocking Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) and 50 µl EasySep™ CD45 Depletion Cocktail were added and incubated at room temperature for 30 minutes. For immunomagnetic labeling EasySep Magnetic Nanoparticles were added at 100 µl/ml and mixed by pipetting. The suspension was incubated at room temperature for 15 minutes and volume was adjusted to 2.5 ml. The tube was placed in an EasySep magnet for 10 minutes. Labeled cells (CD45+) were separated by decanting the supernatant (CD45−) into a new tube. RNA was extracted from the CD45+ and CD45− fractions using the RNeasy® Mini Kit.

Quantitative Reverse Transcription-Polymerase Chain Reaction

RNA samples were quantitated by a NanoDrop™ 2000 spectrophotometer. Subsequently, 0.5 µg RNA was reverse-transcribed using SuperScript® III and random hexamers. The TaqMan® assay was chosen as the quantitative PCR technology to ensure the specificity of detection. PCR reactions were performed on a CFX96 Touch™ Real-Time PCR Detection System. The primers for AR-FL and AR-V7 were previously published.[3] Primers for AR^{v567es} were forward, 5′-CTACTCCGGACCTTACGGGGACATGCG-3′ and reverse 5′-TTGGGCACTTGCACAGAGAT-3′. The probe for all 3 amplicons, which was designed in our laboratory, was 5′-AAGAGCCGCTGAAGGGAAACAGAAGTACTTG-3′. RPL30 (ribosomal protein L30) was used as the housekeeping gene and the assay for RPL30 (Applied Biosystems®) was used. A total of 40 cycles served as the detection limit. Expression levels of the same transcript measured by the 2 approaches were compared using the $2^{-\Delta CT}$ method[15] and adjusted for the difference in starting blood volume.

Subjects

A total of 46 patients with CRPC were selected for study and provided consent via the Tulane Cancer Center

Table 1. *Patient demographics*

	Overall		White		Black	
No. pts	46		41		5	
Median age at diagnosis (range)	60	(43—77)	61.1	(46—77)	57.8	(43—66)
Median total Gleason score (range)	8	(6—9)	8	(6—9)	7	
No. family history (%):						
Yes	12	(26)	11	(27)	1	(20)
No	33	(72)	29	(71)	4	(80)
Unknown	1	(2)	1	(2)		
Median ng/ml PSA at sampling (range)	60.8	(less than 0.01—greater than 3,000)	59.7	(less than 0.01—3,000)	184.4	(48.9—2,050)
Median days diagnosis-sampling (range)	1,877.5	(230—7,781)	1,877.5	(230—7,781)	2,424	(691—7,080)

and/or the urology clinic at Tulane University Hospital. For each patient 5 ml blood were collected in PAXgene tubes. When feasible, serial samples were collected. A total of 73 blood samples were collected and clinically annotated. Table 1 lists patient demographics. Supplementary tables 1 and 2 (http://jurology.com/) show the history of treatment with abiraterone acetate and enzalutamide, and the complete treatment history, respectively. Additionally, blood samples from 21 patients after RP with undetectable PSA (less than 0.01 ng/ml) were analyzed.

Statistical Analysis

All statistical analyses, including descriptive statistics, the Student t-test and the Fisher exact test, were performed with SAS®, version 9.4. All tests were 2-tailed with p values less than the α ($p \leq 0.05$) considered statistically significant. The median and range are reported for all continuous variables and the percent is reported for all categorical variables.

RESULTS

Selection of Whole Blood Approach to AR-V Detection

To identify a protocol for detecting AR-Vs in blood we evaluated the whole blood approach (ie the PAXgene approach) and the CTC enrichment approach based on the depletion of leukocytes (ie the CD45 depletion approach). Ten patients with mCRPC who had progressed to taxane chemotherapy and received multiple rounds of abiraterone and/or enzalutamide were identified for this purpose. Blood samples obtained from the same patient were analyzed side by side (supplementary figure, http://jurology.com/). AR-V7 transcripts were detected in 9 of 10 samples by both methods (supplementary table 3, http://jurology.com/). However, 2 samples were found to be positive for AR^{v567es} by the PAXgene approach but only 1 by the CD45 depletion approach, suggesting that the leukocyte depletion process may lead to loss of sensitivity. Indeed, AR-V transcript levels measured by the CD45 depletion approach were consistently lower than those measured by the PAXgene approach. The estimated decrease in AR-V7 was approximately 40% (supplementary table 4, http://jurology.com/).

The separation of CD45− and CD45+ cells during leukocyte depletion provided an opportunity to investigate the sources of AR transcripts. In 7 of 9 samples the AR-V7 signal was exclusively from the CD45− fraction (table 2). In the remaining samples the levels of AR-V7 measured in the CD45+ fraction were markedly lower than in the CD45− fraction. Similarly, most of the AR^{v567es} transcript was found in the CD45− fraction. In contrast, AR-FL was abundantly expressed in both fractions (table 2). The presence of AR-FL transcripts in CD45+ cells is consistent with studies documenting AR expression in lymphocytes and macrophages.[16,17]

Collectively, these results suggest that the AR-V transcripts in the CD45+ fraction are barely detectable, implying that depleting hematopoietic cells offers little improvement in specificity to detect AR-Vs. Furthermore, performing this procedure could lead to loss of sensitivity.

Whole Blood Assay Sensitivity and Specificity

To assess the sensitivity of this assay 22Rv1 cells, which express AR-V7, were spiked into 5 ml blood from a healthy donor. Figure 1 shows that this assay could detect 5 to 50 AR-V7+ cells in 5 ml blood or 1 to 10 cells per ml.

To evaluate specificity we analyzed blood samples from patients after RP who had undetectable PSA. Neither AR-V7 nor AR^{v567es} was detected in this cohort (table 3). Similarly, AR-V was not detected in

Table 2. *Distribution of AR transcripts in CD45− and CD45+ fractions*

Sample No.	% AR-V7		% AR^{v567es}		% AR-FL	
	CD45−	CD45+	CD45−	CD45+	CD45−	CD45+
1	100.00	0.00	—	—	46.05	53.95
2	100.00	0.00	—	—	29.21	70.79
3	100.00	0.00	—	—	63.96	36.04
4	85.93	14.07	96.16	3.84	55.55	44.45
5	—	—	—	—	65.27	34.73
6	100.00	0.00	—	—	54.75	45.25
7	100.00	0.00	—	—	20.02	79.98
8	91.65	8.35	—	—	76.71	23.29
9	100.00	0.00	—	—	75.59	24.41
10	100.00	0.00	—	—	31.34	68.66

Calculated from expression ratio between 2 fractions with total of 100%.

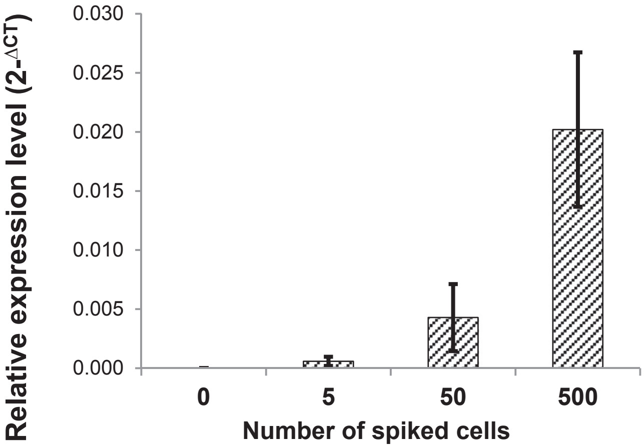

Figure 1. PAXgene assay sensitivity to detect AR-V7. Before transfer to PAXgene tubes 22RV1 cells were spiked into 5 ml blood from healthy donor. RNA extraction and quantitative reverse transcription-PCR analysis were performed as described.

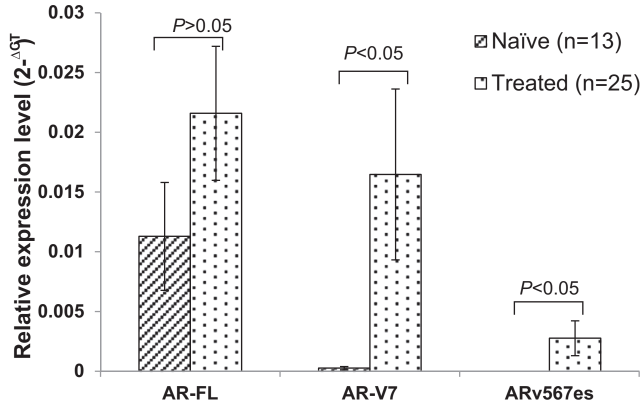

Figure 2. Increased expression of AR-Vs in patients with mCRPC treated with second line hormonal therapy. Naïve patients had never received treatment with abiraterone acetate, ketoconazole or enzalutamide. Treated patients had received these treatments but progressed. Statistical analysis was performed using 2-tailed Student t-test.

blood from 5 healthy donors (data not shown). These results suggest that this assay specifically detects AR-Vs expressed in cancer cells.

AR-V Expression Correlation with Prior Abiraterone and Enzalutamide Treatments

To investigate the clinical relevance of AR-V expression in whole blood we analyzed a total of 73 samples from 46 patients with CRPC. In this cohort 69 samples (94.52%) were positive for AR-FL, 50 (68.49%) were positive for AR-V7 and 23 (31.51%) were positive for AR^{v567es} (table 3). Of the 73 samples 53 (70%) expressed at least 1 variant and 20 (27.40%) expressed both variants. Notably, 20 of the 23 samples that expressed AR^{v567es} were also positive for AR-V7).

Based on the history of second line hormonal therapies, that is abiraterone, ketoconazole and enzalutamide, the CRPC cohort was categorized into naïve and treated groups. Due to a recent report demonstrating an association of AR-V7 expression with prior treatment with docetaxel,[18] patients who had received docetaxel or cabazitaxel were excluded from analysis. The expression level of both variants but not that of AR-FL was higher in the treated group than in the naïve group (fig. 2). In the treated group AR-V7 transcripts were expressed in 17 of 25 samples (68%) compared to 3 of 13 (23.08%) in the naïve group. The Fisher exact test revealed a strong association of AR-V7 positivity with a history of second line hormonal therapies (table 4). Similarly, AR^{v567es} positivity was associated with a history of these therapies, including 9 of 25 treated patients and 0 of 13 naïve patients. Additionally, patients who had been treated with second line hormonal therapies were more likely to express 1 (AR-V+) or both (AR-V7+/AR^{v567es}+) AR-V isoforms.

DISCUSSION

As noted by many groups, the development of predictive biomarkers is critical to optimal clinical decision making. The study by Antonarakis et al clearly represents a step forward in this direction.[7] Based on this ground breaking study the AR-V7 transcript in CTCs predicts resistance to abiraterone and enzalutamide. However, a potential shortcoming of this assay is dependence on the selection

Table 3. *AR transcript detection in 2 cohorts*

	Post-RP		mCRPC		p Value	
Median age at sampling (range)	65.05 (53–76)		68.52 (47–86)		0.1559	(Student t-test)
No. samples (%):	21		73		—	
AR-FL+	17	(80.95)	69	(94.52)	0.0712	(Fisher exact test)
AR-V7+			50	(68.49)	<0.0001	(Fisher exact test)
AR^{v567es}+	0		23	(31.51)	0.0014	(Fisher exact test)
AR-V+	0		53	(72.60)	<0.0001	(Fisher exact test)
AR-V7+, AR^{v567es}+	0		20	(27.40)	0.0051	(Fisher exact test)

Table 4. *AR-V positivity correlated with prior history of second line hormonal treatments*

	No. Naïve	No. Treated	p Value (Fisher exact test)
Overall	13	25	—
AR-FL+	12	24	1.0000
AR-V7+	3	17	0.0156
AR^{v567es}+	0	9	0.0159
AR-V+	3	18	0.0062
AR-V7+/AR^{v567es}+	0	8	0.0335

of EpCAM+ or Her2+ CTCs,[19] which likely represent only a fraction of the entire CTC population. In addition, the use of an epithelial marker such as EpCAM may exclude the population that underwent EMT. This is particularly relevant since studies have shown that AR-V7 and AR^{v567es} promote EMT in prostate cancer cells,[20–23] raising concern that AR-V expressing CTCs may not be captured efficiently by epithelial markers. Furthermore, it is clear from studies of CTCs using immunodetection methodologies that not all patients have detectable CTCs,[24] thus, limiting the number of patients in whom these AR-V7 assays can be performed.

To avoid the problems associated with CTC positive selection we tested a whole blood based approach and a negative selection approach by depleting lymphocytes. A comparison of these approaches led us to conclude that the whole blood approach performed at least as well as the CD45 depletion approach with regard to the sensitivity of AR-V detection (supplementary table 3, http://jurology.com/). The expression of AR-Vs was found predominantly in the CD45− population (table 2), suggesting that depleting CD45+ cells is unnecessary. Furthermore, the variants were detected at lower levels by the CTC enrichment protocol (supplementary table 4, http://jurology.com/), possibly due to the lack of RNA preservation during the enrichment process.

In a few cases we detected variants in the CD45+ fraction (table 2). This was most likely a result of cross contamination rather than expression of the variants by CD45+ cells. This is supported by data on the post-RP cohort showing that neither AR-V was detectable in whole blood derived RNA (table 3). Cross contamination could be caused by nonspecific binding of the CD45 antibodies, aberrant expression of CD45 by cancer cells[25,26] or the formation of lymphocyte/cancer cell microemboli.[27] Regardless, this observation suggests that CTCs could be lost during negative selection.

Recently, Steinestel et al reported using AR-V7 and AR mutations in CTCs to guide a therapy switch in patients.[18] The estimated overall benefit of the molecularly informed treatment decision was 27% over the uninformed decision. This study along with that of Antonarakis et al[7] highlights the importance of incorporating AR-V profiling into individualized treatment decision making. Although our assay is not complement-dependent cytotoxicity based, the findings are in line with complement-dependent cytotoxicity based studies, supporting a role of AR-V7 expression in resistance to second line hormonal therapies.

The current findings have several limitations. 1) This is a cross-sectional rather than a prospective study. A prospective study with longitudinal evaluation of patients is needed to establish the expression of AR-Vs in whole blood as a biomarker of treatment responsiveness. 2) Cells expressing the AR transcripts are not assessable by this assay. As a result some questions could not be properly addressed, such as whether AR-FL and AR-Vs are coexpressed in the same cells. These questions are clinically significant because AR-Vs have been shown to heterodimerize with AR-FL[28] and facilitate its nuclear translocation.[29] 3) There is no tissue confirmation using direct biopsies of tumors.

CONCLUSIONS

Despite the shortcomings, the current study is novel. To our knowledge this is the first report of AR-V7 detection using a whole blood assay that also detected AR^{v567es} in a significant proportion of patients. Eliminating the CTC selection process decreases hands-on time and improves assay sensitivity, enabling more patients to benefit from such assays. Indeed, the percent of AR-V7+ patients in this study was higher than for both CTC based assays (68.49% vs 50% and 49%, respectively).[7,18] The small volume of blood needed and the stability of RNA in PAXgene tubes make it practical to incorporate this assay into routine patient monitoring. In view of the potential benefits it is necessary to further evaluate and refine this assay in a prospective study with an adequate cohort.

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